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The Middle Region of an HP1-binding Protein, HP1-BP74, Associates with Linker DNA at the Entry/Exit Site of Nucleosomal DNA*[S]

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In higher eukaryotic cells, DNA molecules are present as chromatin fibers, complexes of DNA with various types of proteins; chromatin fibers are highly condensed in metaphase chromosomes during mitosis. Although the formation of the metaphase chromosome structure is essential for the equal segregation of replicated chromosomal DNA into the daughter cells, the mechanism involved in the organization of metaphase chromosomes is poorly understood. To identify proteins involved in the formation and/or maintenance of metaphase chromosomes, we examined proteins that dissociated from isolated human metaphase chromosomes by 0.4 M NaCl treatment; this treatment led to significant chromosome decondensation, but the structure retained the core histones. One of the proteins identified, HP1-BP74 (heterochromatin protein 1-binding protein 74), composed of 553 amino acid residues, was further characterized. HP1-BP74 middle region (BP74Md), composed of 178 amino acid residues (Lys^{97}–Lys^{274}), formed a chromatosome-like structure with reconstituted mononucleosomes and protected the linker DNA from micrococcal nuclease digestion by ~25 bp. The solution structure determined by NMR revealed that the globular domain (Met^{153}–Thr^{237}) located within BP74Md possesses a structure similar to that of the globular domain of linker histones, which underlies its nucleosome binding properties. Moreover, we confirmed that BP74Md and full-length HP1-BP74 directly binds to HP1 (heterochromatin protein 1) and identified the exact sites responsible for this interaction. Thus, we discovered that HP1-BP74 directly binds to HP1, and its middle region associates with linker DNA at the entry/exit site of nucleosomal DNA *in vitro*.

In eukaryotic cells, genomic DNA associates with various types of proteins to form chromatin fibers. During mitosis, the chromatin fibers undergo drastic structural changes to organize the metaphase chromosomes, which ensures the equal and appropriate segregation of genomic information into the daughter cells. Although the process of chromosome organization was first described by Flemming in 1882 (1) and the chromosome structure has been investigated by numerous researchers (2), the mechanisms involved in chromatin condensation and the structure of the metaphase chromosome are still poorly understood (3). Previously, we successfully determined the overall protein composition of human metaphase chromosomes through proteome analysis (4, 5). We classified the identified proteins into four different groups based on their localization and biochemical properties: chromosome structural proteins, chromosome peripheral proteins, chromosome fibrous proteins, and chromosome coating proteins (5). Proteins contributing to the formation of chromosome structure belong to the chromosome structural protein group.

The proteins involved in chromosome organization can be further divided into two different groups, according to how they contribute to the formation and/or maintenance of chromosome structure, namely proteins responsible for organizing chromosome structure and proteins that play roles in the regulation of chromatin structure. One of the well known proteins in the former group is condensin. Condensin is a protein complex composed of two SMC (structure maintenance of chromosome) subunits and three non-SMC subunits (6). In vertebrates, two different condensin complexes, condensin I and II, have been identified (6–8). Several studies have shown that the condensin complexes are required for the structural integrity of mitotic chromosomes and their proper segregation; on the other hand, mitotic chromosomes that lack condensins surprisingly exhibit almost normal condensation (6, 7, 9, 10). Topoisomerase IIα is another molecule that has been studied as a condensation factor of mitotic chromosomes. Topoisomerase IIα is localized to the axial region of mitotic chromosomes, similar to condensin (11). However, knockdown of topoisomerase IIα caused partial chromosome segregation failure but did not result in condensation defects (12–14). On the other hand, several other factors, such as high mobility groups, poly(ADP-ribose) polymerase, HP1s (heterochromatin proteins 1), and

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The atomic coordinates and structure factors (code 2rqp) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S6.

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linker histones, were reported to be involved in the regulation of chromatin structure (15). Of these, the linker histone H1 family is the most extensively studied. The globular domain (GD)[3] of H1 associates with linker DNA at the entry/exit site of nucleosomal DNA, and the C-terminal region interacts with linker DNA to form a "stem" structure (15). The structure formed by the association of H1 with the nucleosome is called the chromatosome, which is considered to be the basic repeat unit of the chromatin fiber. It is generally believed that H1 is involved in mitotic chromatin condensation; however, the role of H1 in this process is rather controversial. In earlier studies, it was reported that H1 is not required for mitotic chromosome condensation because condensation can occur in H1-depleted systems (16, 17). However, more recently, it was shown that H1 plays an important role in the condensation and segregation of vertebrate chromosomes in mitosis (18).

In this study, to identify proteins involved in the formation and/or maintenance of the metaphase chromosome, we determined the NaCl concentrations at which the morphology of the isolated metaphase chromosomes dramatically changes and identified the proteins that dissociated from the chromosomes at that NaCl concentration. One of the identified proteins, HP1-BP74 (heterochromatin protein 1-binding protein 74), was further characterized. HP1-BP74 was identified in yeast two-hybrid screens as a mouse HP1α partner and is known to have significant primary structure similarity to the GD of the linker histone family (19). However, its function remains to be investigated. Here, we demonstrate that a globular domain (BP74GD) located in the middle region of HP1-BP74 (BP74Md) has a linker histone-like structure and that BP74Md binds to nucleosomes and protects extra DNA from MNase digestion; moreover, BP74Md interacts with HP1 *in vitro* through a newly identified binding site located at its unstructured region. HP1s are well known proteins that contribute to the regulation of chromatin structure (20). Thus, we suggest that HP1-BP74 could play a role in the formation and/or maintenance of the compacted chromatin structure through HP1 proteins binding and possibly also through its middle part, BP74Md, which associates with linker DNA at the entry/exit site of nucleosomal DNA.

EXPERIMENTAL PROCEDURES

Cell Culture—HeLa S3 cells were maintained in RPMI1640 medium supplemented with 5% (v/v) fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Both cell lines were grown at 37 °C and 5% CO_2 in a humidified incubator.

Isolation of Human Metaphase Chromosomes—Human metaphase chromosomes were prepared from HeLa S3 cells according to a previously described procedure (21). Briefly, cells synchronized with 0.1 μg/ml colcemid for 16 h were hypotonically treated and homogenized in polyamine buffer containing 15 mM Tris-HCl (pH 7.2), 2 mM EDTA, 80 mM KCl, 20 mM NaCl, 0.5 mM EGTA, 0.2 mM spermine, 0.5 mM spermidine, 0.1% 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 0.1% digitonin. Metaphase chromosomes were collected in the supernatant by centrifugation for 3 min at 440 $\times$ *g* at 4 °C and concentrated in isolation buffer containing 5 mM Tris-HCl (pH 7.4), 20 mM KCl, 20 mM EDTA, 0.25 mM spermidine, 1% thiodiglycol, 0.1 mM phenylmethylsulfonyl fluoride, 0.1% empigen, and 70% glycerol by centrifugation for 20 min at 1,750 $\times$ *g* at 4 °C. The chromosome fraction was further purified using Percoll density gradient centrifugation according to the method previously developed by Gasser and Laemmli (22). The purified metaphase chromosomes were concentrated by centrifugation for 15 min at 3,000 $\times$ *g* at 4 °C.

Salt Stripping of the Isolated Metaphase Chromosomes—The isolated metaphase chromosomes were dropped onto a coverslip and a small piece of Parafilm was placed on the drop to spread the chromosomes across the coverslip. After a 10-min incubation on ice, the sample was washed with isolation buffer for 5 min, followed by incubation in XBE2 buffer (10 mM HEPES (pH 7.7), 2 mM $MgCl_2$, 100 mM KCl, and 5 mM EGTA) containing NaCl at different concentrations for 1.5 h on ice. Following the incubation, the samples were fixed with 2% paraformaldehyde in XBE2 buffer (containing the appropriate NaCl concentration) for 15 min at room temperature, followed by staining with 1 μg/ml 4′,6-diamidino-2-phenylindole.

Identification of the Dissociated Proteins—Chromosomes are highly sticky to each other, and once they stick together, most of the chromosome proteins are not accessible to the solvent. Therefore, dissociation of chromosome proteins at 0.4 M NaCl was carried out repeatedly in dilute solutions. The metaphase chromosomes were diluted more than 8-fold in XBE2 buffer and subsequently centrifuged in the XBE2 buffer containing 70% (w/v) glycerol and 0.4 M NaCl for 15 min at 3,000 $\times$ *g* at 4 °C. The fraction containing chromosomes was mixed with 3 volumes of XBE2 buffer containing 0.4 M NaCl, followed by incubation for 1.5 h at 4 °C. After centrifugation for 30 min at 17,400 $\times$ *g* at 4 °C, the supernatant was collected to identify the dissociated proteins. Proteins from the supernatant were trichloroacetic acid-precipitated and applied to 12 or 5–10% SDS-PAGE, and visualized by Coomassie Brilliant Blue (CBB) staining. Individual bands were excised from the gel, subjected to in-gel trypsin digestion, and identified by mass spectrometry.

Plasmid Constructions—The HP1-BP74 cDNA sequence was amplified from a HeLa cDNA library (human HeLa large insert cDNA library, Clontech) using specific primers for the open reading frame of the gene, 5′-GGTACTAGTATGGCGACTGATACGTCTCAAGG-3′ and 5′-ACCGTCGACCTTTTTCACTCTGAAAGACTTC-3′; a SpeI linker was added to the 5′-primer, and a SalI linker was added to the 3′-primer. Synthesized cDNAs were digested with SpeI and SalI and then cloned into the pIC113 vector. To generate a pEGFP/HP1-BP74 plasmid, full-length cDNA was amplified by PCR from pIC113/HP1-BP74 and cloned into pEGFP-C3 vector at XhoI and SalI sites. DNA fragments of BP74Md (Lys^{97}–Lys^{274}) and BP74GD (Met^{153}–Thr^{237}) were amplified by PCR from full-length cDNA and cloned into pET48b at SmaI and EcoRI sites and XmaI and EcoRI sites, respectively. Plasmids for HP1α mutants were generated from pET15b-HP1α, a kind gift from Prof. D. J. Tremethick (23). The primers used to generate the HP1α C133A

[3] The abbreviations used are: GD, globular domain; CBB, Coomassie Brilliant Blue; GFP, green fluorescent protein; CSD, chromo shadow domain.

mutation were 5′-GCAACAGATTCCGCCGGTGATTTA-ATG-3′ and 5′-CATTAAATCACCGGCGGAATCTGTTGC-3′. The primers used to generate the HP1α W174A mutation were 5′-GAGACTGACAGCGCATGCATATCC-3′ and 5′-GGATATGCATGCGCTGTCAGTCTC-3′.

Production of Recombinant Proteins—A Trx-His-tagged recombinant BP74GD fusion protein was expressed in *Escherichia coli* Rosetta (DE3) pLysS. Cells were grown to $A_{600} = 0.6$ at 37 °C in 1 liter of LB medium. Then, after further incubation for 2 h at 37 °C with 1 mM isopropyl 1-thio-β-D-galactopyranoside, the cells were harvested and lysed in 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and Complete protease inhibitor mixture (Roche Applied Science). Trx-His-tagged proteins were purified from the supernatant by affinity chromatography using a HisTrap column (GE Healthcare). The tag was cleaved by incubation with HRV3C (Novagen) for 36 h at 4 °C in 50 mM Tris-HCl buffer (pH 8.0) containing 500 mM NaCl and removed by the HisTrap column. The desired proteins in the flow-through were further purified by gel filtration chromatography (GE Healthcare) in 50 mM sodium phosphate (pH 6.0), 150 mM NaCl, and 5 mM 2-mercaptoethanol. BP74Md was expressed and purified using a similar procedure to BP74GD, except for the additional steps described below between the first affinity purification and the tag cleavage; after affinity purification, the Trx-His-tagged proteins were mixed with polyethyleneimine at a final concentration of 1% and centrifuged. The Trx-His-tagged proteins were precipitated from the obtained supernatant by 40% ammonium sulfate saturation and dissolved in 50 mM Tris-HCl (pH 8.0), followed by ion exchange chromatography using a SP HiTrap column (GE Healthcare).

His-tagged recombinant HP1α proteins were expressed under the same conditions as BP74GD. The harvested cells were lysed in 10 mM Tris-HCl (pH 7.4), 300 mM NaCl, 0.1 mM EDTA and Complete protease inhibitor mixture (Roche Applied Science). His-tagged proteins were purified from the supernatant by affinity chromatography using a HisTrap column (GE Healthcare), and purified by ion exchange chromatography using an SP HiTrap column at pH 5.0 and gel filtration chromatography.

Nucleosome Binding Assay (Gel Shift and Chromatosome Stop)—Mononucleosomes were reconstituted from chicken core histone octamers and 208-bp DNA. The 208-bp DNA was prepared by digesting p208-35 plasmid, a derivative of pPol1208 (24), with AvaI (New England Biolabs) and separating the DNA fragments on a Sephacryl S500 (GE Healthcare) gel filtration column. Histone octamers were purified from chicken erythrocytes (Pel Freeze, Rogers, AR) using hydroxyapatite chromatography (25) and analyzed on SDS-PAGE. Mononucleosome reconstitution was performed using a previously described method (26). The reconstituted mononucleosomes were incubated with hH1.2 (purchased from BIOMOL GmbH) or BP74Md for 1 h at room temperature in 10 mM HEPES (pH 7.5), 50 mM KCl, 5 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 5% glycerol. For gel shift, the reactants were analyzed on 0.7% agarose gel in 0.5× TBE, followed by SYBR Green I staining. For the chromatosome stop, 0.2 units of micrococcal nuclease (MNase) were added per 1 μg of DNA and incubated in the presence of 1 mM $CaCl_2$. Digestion was stopped by introducing 6 mM EDTA and 0.4% SDS and placing the tube on ice for 10 min. Proteinase K was then added to a final concentration of 100 ng/μl, and the sample was incubated for 1 h at 37 °C. DNA was phenol-extracted and ethanol-precipitated, and the pellet was dissolved in TE buffer (pH 7.5) and analyzed on 15% polyacrylamide gel in 1× TBE, followed by SYBR Green I staining.

NMR Spectroscopy—Uniformly ^{15}N- and ^{13}C-labeled BP74GD was overexpressed by growing *Escherichia coli* BL21 (DE3) cells in M9 minimal medium containing [^{15}N]ammonium chloride (1 g/liter) and/or [^{13}C]glucose (2 g/liter) as the sole nitrogen and carbon sources. The NMR samples were prepared in 50 mM sodium phosphate, 150 mM NaCl, and 5 mM 2-mercaptoethanol in 100% D_2O or a 90% H_2O, 10% D_2O mixture at pH 6.0. The protein concentration was adjusted to ~0.5 mM in a 5-mm microcell NMR tube (Shigemi) for all NMR studies.

The backbone and side chain ^1H, ^{15}N, and ^{13}C resonances of BP74GD were assigned by standard double and triple resonance NMR experiments. Sequence-specific backbone assignments were achieved by two-dimensional ^1H-^{15}N heteronuclear single quantum correlation (HSQC) and three-dimensional HNCO, HN(CA)CO, CBCA(CO)NH, and HNCACB spectra. Assignments of side chain resonances were obtained from two-dimensional ^1H-^{13}C HSQC and three-dimensional HBHA(CO)NH, H(C)CH correlation spectroscopy (COSY), H(C)CH-total correlated spectroscopy (TOCSY), and (H)CCH-TOCSY. Nuclear Overhauser effects were collected from three-dimensional ^{15}N-edited nuclear Overhauser effect spectroscopy (NOESY) (100-ms mixing time) and ^{13}C-edited NOESY (100-ms mixing time) spectra (27, 28). The backbone amide groups that slowly exchanged with the solvent were identified from a series of two-dimensional ^1H-^{15}N HSQC spectra following a rapid buffer exchange to D_2O. All NMR data were processed with NMRPipe (29) and analyzed with NMRVIEW (Merck Research Laboratories).

Structure Calculation—Nuclear Overhauser effect restraints were classified into four categories: strong, medium, weak, and very weak, corresponding to the distance restraints of 1.8–2.8, 1.8–3.4, 1.8–4.2, and 1.8–5.0 Å, respectively. The ψ and φ torsion angle restraints were evaluated from the ^{15}N, Hα, $^{13}C\alpha$, and $^{13}C\beta$ chemical shifts using the TALOS program (30). The restraints deduced from the intramolecular hydrogen bonds of protein backbone, which were identified by hydrogen-deuterium exchange experiments, were classified into two groups: between the amide proton and the carbonyl oxygen of 1.5–2.5 Å and between the amide nitrogen and the carbonyl oxygen of 2.5–3.5 Å (31). The initial solution structures were calculated using the distance geometry algorithm in the CNS programs (32). Structural optimization and energy minimization were achieved by a simulated annealing algorithm. The final 12 lowest energy structures were analyzed using the MOLMOL (33) and PROCHECK programs (34). Structural statistics for the 12 structures are included in supplemental Table S1. Graphical representations were prepared using PyMOL (available on the World Wide Web).

Localization Analysis—pEGFP/HP1-BP74 was transfected into HeLa cells using FuGENE6 (Roche Applied Science).

Observation was performed 24 h posttransfection. A stable cell line expressing pEGFP/H1.2 was obtained as previously described (35). Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and stained with Hoechst 33342.

Pull-down Assay—30 μl of nickel-Sepharose 6 Fast Flow beads (GE Healthcare) were equilibrated with PD buffer, 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 100 mM imidazole, and 1% Triton X-100. The beads were mixed with 100 pmol of recombinant His-tagged HP1α dimers in 200 μl of PD buffer, followed by incubation with gentle agitation for 2 h at 4 °C. The mixture was centrifuged at 500 × *g* for 5 min, and the precipitant was washed twice with 200 μl of PD buffer. Recombinant BP74Md or BP74GD in 200 μl of PD buffer (2.5-fold molar excess over HP1 dimers) was added to the precipitant, followed by incubation with gently agitation for 16 h at 4 °C. The mixture was centrifuged at 500 × *g* for 5 min, and the precipitant was washed five times with 200 μl of PD buffer. Then, 2× SDS sample buffer was added to the precipitant, boiled for 5 min, and analyzed by SDS-PAGE with CBB staining.

Interaction Assay between Endogenous Full-length HP1-BP74 and Recombinant HP1α—Chromosomal proteins containing endogenous HP1-BP74 were prepared by 0.4 M NaCl stripping as described under "Identification of the Dissociated Proteins" but in buffer without EGTA. The solution was diluted with the buffer without NaCl to adjust the concentration of NaCl to 150 mM and concentrated. The solution was employed for a pull-down assay with 1 nmol of recombinant His-tagged HP1α dimers in 200 μl of PD buffer.

Antibodies—Anti-HP1α antibody (MAB3446) was purchased from Millipore. Anti-HP1-BP74 antibody was produced by immunizing a rabbit with recombinant protein containing N-terminal 118 amino acids of HP1-BP74 (this sequence is highly specific to HP1-BP74). The produced polyclonal antibody was affinity-purified using the recombinant antigen protein.

RESULTS

Isolated Metaphase Chromosomes Show Swollen Morphology, and Histone H1 Proteins Dissociate in the Presence of 0.4 M NaCl—In our previous proteome analysis of human metaphase chromosomes, we identified over 200 human chromosomal proteins (4, 5). Although we successfully identified the protein complement of the metaphase chromosomes, it is obvious that not all of the proteins are involved in chromosome structure organization. In order to identify proteins that play a role in the formation and/or maintenance of chromosome structure, we performed salt stripping of proteins from isolated chromosomes. Salt stripping is an approach in which proteins are divided into different populations on the basis of their electrostatic affinity to chromosomes. Previously, Paulson and Laemmli (36) used 2 M NaCl to dissociate proteins, including core and linker histones, from isolated metaphase chromosomes and referred to the remaining structure as the chromosome scaffold. Topoisomerase IIα and one of the condensin subunits (ScII) were identified as components of the chromosome scaffold (37, 38). Later, Earnshaw and co-workers (39) reported the

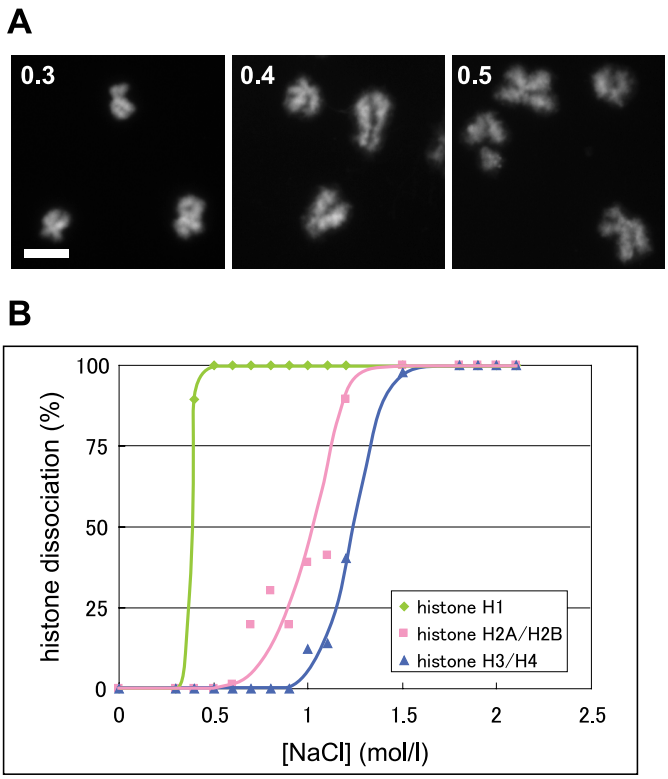

FIGURE 1. **NaCl stripping of metaphase chromosomes isolated from HeLa S3 cells.** *A*, morphology of the chromosomes at the indicated NaCl concentrations (mol/liter). The chromosomes were stained with 4′,6-diamidino-2-phenylindole. *Bar*, 10 μm. *B*, dissociation rates of linker and core histones under different concentrations of NaCl. Histone dissociation (%) was calculated by comparing the band intensities between the dissociated fraction and the chromosome-bound fraction.

protein composition of the chromosome scaffold, also focusing on the proteins remaining after 2 M NaCl treatment. In the present study, we focused on proteins with weaker affinity that seem to be involved in the organization of chromosome structure.

Throughout this analysis, we used highly purified human metaphase chromosomes obtained using a newly modified method that eliminates contaminant proteins, such as chromosome coating proteins (21); thus, we investigate only *bona fide* chromosome proteins, such as chromosome structural proteins and chromosome peripheral proteins (5, 21). First, we observed the effect of salt concentration on the morphology of chromosomes. The chromosomes isolated from HeLa S3 cells were mounted on poly-L-lysine-coated coverslips and gently incubated in buffer containing various concentrations of NaCl (Fig. 1*A*). After treatment with 0.4 M NaCl, the size of the isolated metaphase chromosomes was significantly larger than that of chromosomes incubated at concentrations less than 0.3 M NaCl. DNA haloes appeared around the chromosomes, and sister chromatids separated at the arm regions. The sizes of chromosomes treated with 0.5 or 0.6 M NaCl did not dramatically increase compared with those at 0.4 M NaCl. These results suggested that 0.4 M is the critical NaCl concentration at which the morphology of the chromosomes dramatically changes.

It has been reported that linker histones dissociate from calf thymus chromatin around this NaCl concentration (40). Therefore, we analyzed the affinity of the core and linker his-

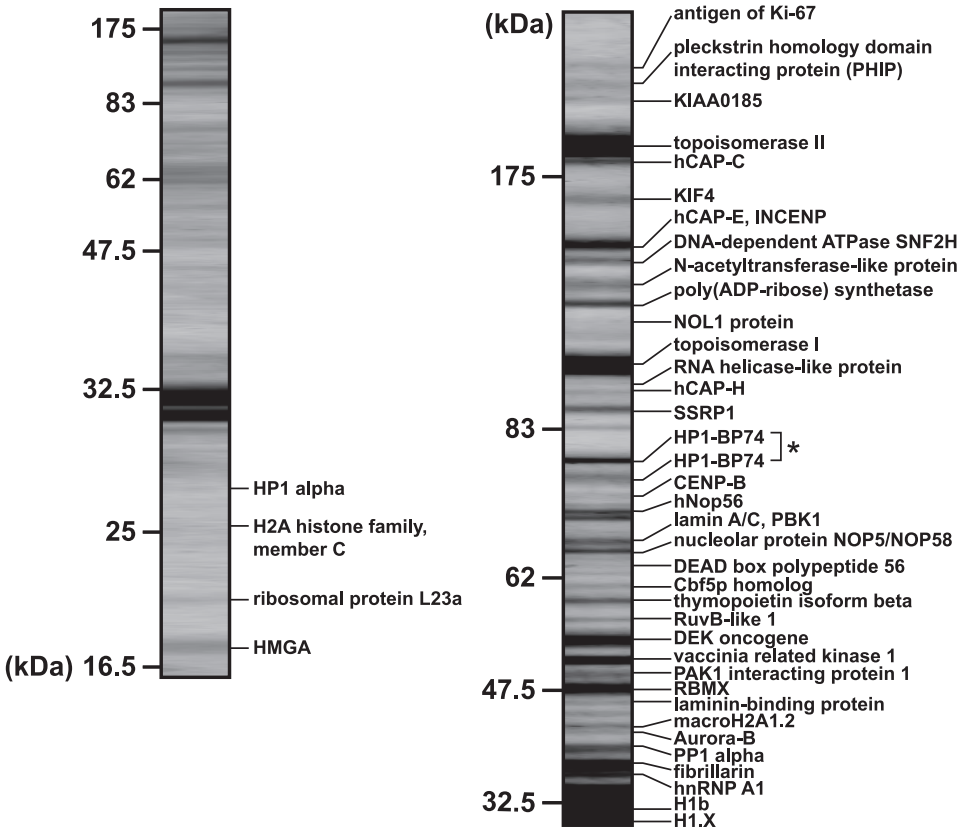

FIGURE 2. **Identification of the 0.4 M NaCl-dissociated proteins.** The dissociated proteins were separated by SDS-PAGE on 12% (*left*) and 5–10% (*right*) polyacrylamide gels, followed by CBB staining. The bands were then excised for protein identification by mass spectrometry.

tones for the isolated metaphase chromosomes at different NaCl concentrations. The isolated chromosomes were incubated in buffer containing the appropriate concentration of NaCl followed by the separation of the dissociated proteins by centrifugation. The proteins in the supernatant or the precipitant were resolved on polyacrylamide gels and visualized by CBB staining, and the dissociation ratios of each histone were determined by comparing the band intensities between the supernatant ("dissociated" fraction) and the precipitant ("chromosome-bound" fraction) (Fig. 1*B*). Similar to the chromatin study (40), at 0.4 M NaCl, the H2A/H2B dimers and the H3/H4 tetramers remained on the chromosomes; ~90% of the linker histone H1s had dissociated from the chromosomes. We then identified by mass spectrometry all 0.4 M NaCl-dissociated proteins that were present as CBB-stained bands on polyacrylamide gels (Fig. 2). Several well known chromosomal proteins, including linker histones, topoisomerase IIα, and high mobility groups were among the 42 proteins identified.

BP74Md Binds to Nucleosomes and Protects Extra Linker DNA—We selected HP1-BP74 for further characterization because this protein was detected in relatively large amounts (Fig. 2, *asterisk*). HP1-BP74 constitutes from 553 amino acids and was also identified as a chromosomal protein in every cell line we analyzed, as shown in our previous proteome analyses (4, 5), yet no functional study in either interphase or mitotic phase has been reported. HP1-BP74 was identified from the two different bands (Fig. 2, *asterisk*), with the amount of the lower one smaller than that of the higher. This suggests possible different posttranslational modifications or splicing variants. HP1-BP74 was first identified as a binding partner of HP1α in yeast two-hybrid screens for mouse HP1α partners (19). BP74Md (K97-K274) include BP74GD (M153-T237) that has sequence similarity to the GD of linker histones (Fig. 3*A*) (19). No putative function was predicted from the primary structure of the rest of the molecule. The amino acid sequences of N-terminal and C-terminal regions suggest that both regions have intrinsically disordered structure (supplemental Fig. S1).

We investigated whether recombinant BP74Md binds to the nucleosome and linker DNA to form a chromatosome-like structure, as H1 does. The extremely low expression level and/or insolubility of the full-length recombinant HP1-BP74 precluded its preparation; thus, we expressed and purified BP74Md comprising 178 amino acids from Lys^{97} to Lys^{274} (see Fig. 3*A*). This region contains an amino acid sequence, BP74GD (Met^{153}–Thr^{237}), similar to that of the GD of the linker histones. Mononucleosomes were reconstituted by a salt dialysis method, using 5 S ribosomal DNA 208 bp in length and core histone octamers prepared from chicken erythrocytes. First, the reconstituted mononucleosomes were incubated with BP74Md or full-length human H1.2 as a control and analyzed on an agarose gel (Fig. 3*B*). Incubation with increasing amounts of H1.2 led to the formation of a larger complex, which finally aggregated and remained in the well of the agarose gel (Fig. 3*B*, *lane 6*). The mixture of mononucleosomes and BP74Md also showed a mobility shift on the gel.

To further investigate the binding of BP74Md on the nucleosome, the mixture of the reconstituted mononucleosomes and BP74Md was subjected to MNase digestion. The DNA was then purified and analyzed for the presence of the so-called "chromatosome stop" (Fig. 3*C*). The addition of excess H1.2 or BP74Md produced not only chromatosomes but also larger complexes, as shown in *lanes 4–6* and *10–12*. We further analyzed in detail the mixtures shown in *lanes 2* and *9* in Fig. 3*B*. As shown in Fig. 3*C*, when the mononucleosome alone was exposed to MNase digestion, the 208-bp DNA was digested to ~150-bp DNA fragments corresponding to the length of DNA in the nucleosome core particle. As expected, the addition of H1.2 results in the multiple bands corresponding to intermediates (Fig. 3*C*), indicating that H1.2 protects extra DNA against MNase digestion, as reported previously (41). For BP74Md, although the length of the protected fragment was a little shorter than that with H1.2, 21 and 27 bp of extra protection was clearly detected. This additional protection (over that

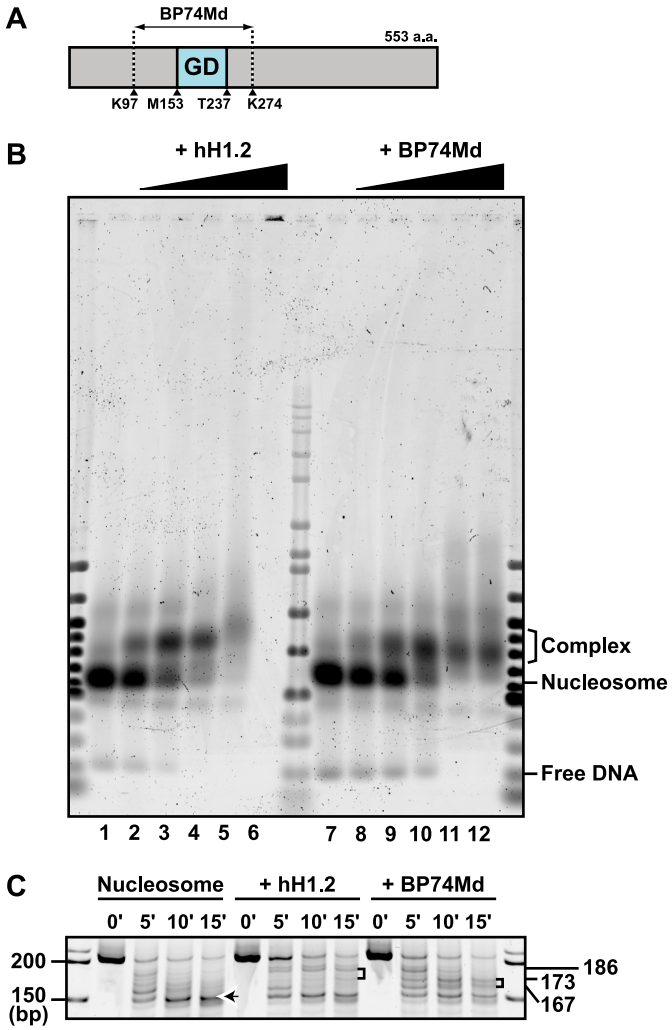

FIGURE 3. **Nucleosome binding activity of BP74Md *in vitro*.** *A*, schematic figure of full-length HP1-BP74. The BP74GD is shown in a *light blue box*. *B*, analysis of nucleosome binding activity of BP74Md and hH1.2 as a control by gel shift assay. Mononucleosomes (0.3 μM) were reconstituted using core histone octamers prepared from chicken erythrocytes and 208-bp DNA and incubated with 0, 0.15, 0.3, 0.6, 0.9, or 1.2 μM hH1.2 or BP74Md (*lanes 1–6* and *7–12*, respectively). The positions of free DNA, nucleosomes, and complex are indicated, as is the complex formed as a result of hH1.2 or BP74Md binding. *C*, chromatosome stop assay. Mononucleosomes alone as a control or a mixture of mononucleosomes and hH1.2 or BP74Md (samples from *lanes 2* and *9* in Fig. 3*B*, respectively) were digested with 0.2 units of MNase for 0, 5, 10, or 15 min at room temperature. The *arrow* and *brackets* show DNA fragments protected within the nucleosome or a nucleosome-protein complex, respectively.

endowed by the core histones) indicates that BP74Md binds at the entry/exit site of the nucleosomal DNA.

Solution Structure of BP74GD and BP74Md—We measured the CD spectra to estimate the secondary structures of BP74GD and BP74Md (supplemental Fig. S2). The CD spectra indicated that BP74GD was α-helix-rich, whereas the α-helix content in BP74Md was reduced; no significant spectrum of β-sheet was obtained, suggesting that α-helix formation is limited to the GD and that the other region in BP74Md is unstructured. We next determined the three-dimensional solution structure of BP74GD by NMR. Uniformly ^{15}N- and ^{13}C-labeled BP74GD gave a well resolved ^{1}H-^{15}N HSQC spectrum (data not shown), suggesting that the protein was stably folded. Assignments of the backbone resonances and the side chain resonances were successfully carried out, and the experimental restraints required for the structural calculation were obtained. The root mean square deviation of 12 resultant structures with the lowest energy of the target functions was 0.31 $\pm$ 0.04 Å for backbone atoms and 0.91 $\pm$ 0.09 Å for heavy atoms in the regular secondary structure elements (residues 13–21, 31–41, 43–50, and 53–64). PROCHECK-NMR (34) analysis showed that 86.4 and 13.6% of the backbone angles lay in regions of Ramachandran space classified as most favored and additionally allowed, respectively (supplemental Table S1). The structure closest to the average of the grouped resultant structures is shown in Fig. 4*A*. The solution structure of BP74GD is very similar to the linker histone GD, supporting its possible linker histone-like function on nucleosomes. Of note, the region containing the α-helices of BP74GD showed a high similarity with those of chicken H1 (42) and H5 (43), with ~1.5 Å root mean square deviation. BP74GD consists of four α-helices, whereas chicken H1 possesses three α-helices and a β-sheet. The additional α-helix composed of Pro^{192}–Arg^{199}, corresponding to a loop region in chicken H1, contains more amino acids than the loop region of H1, contributing to the stabilization of α-helix formation. This α-helix composed of Pro^{192}–Arg^{199} contains a P*X*L*X*L sequence (supplemental Fig. S3*A*), which was reported to be responsible for HP1 binding (44).

GFP-fused HP1-BP74 Is Co-localized with Chromatin throughout the Cell Cycle—As described above, we demonstrated that BP74Md possesses structural and some functional similarities with the GD of linker histones. To characterize the full-length HP1-BP74 *in vivo*, the localization of N-terminal GFP fusion protein transiently expressed in HeLa cells was investigated (Fig. 5). GFP/HP1-BP74 was localized in nuclei but not in nucleoli in interphase and colocalized with chromosomes in mitosis. These localization patterns are generally observed for proteins that are involved in the formation of fundamental structure in chromatin, such as core and linker histones and high mobility groups (45).

HP1-BP74 Binds to HP1α through a Novel PXVXL Motif in Vitro—HP1 proteins form homodimers through a certain isoleucine on the chromo shadow domain (CSD) (Ile^{165} in human HP1α). This dimer interacts with its binding partners having the consensus amino acid sequence PXVXL and its variants (44, 46–48). Structural analysis of the complex of mouse HP1β CSD and a CAF-1 (chromatin assembly factor-1) peptide showed that Trp^{170} on CSD (Trp^{174} in human HP1α) plays a central role in recognizing the PXVXL motif that exists in its binding partners (47). In fact, substitution of the tryptophan to an alanine residue in CSD disrupts the interaction with its binding partners while still allowing formation of the HP1 dimer (44, 46, 47).

To investigate whether HP1-BP74 binds to HP1α, we performed a pull-down assay using His-tagged recombinant proteins. Of note, the recombinant HP1α proteins used for this assay had a Cys to Ala substitution (C133A), because wild-type HP1α rapidly forms higher oligomers by disulfide bond formation (supplemental Fig. S4). BP74Md co-precipitated with His-tagged HP1α^{C133A}, but not with His-tagged HP1$\alpha^{C133A/W174A}$ (Fig. 6*A*), suggesting that HP1α binds to BP74Md *in vitro* through Trp^{174}-P*X*V*X*L motif interactions. The P*X*L*X*L

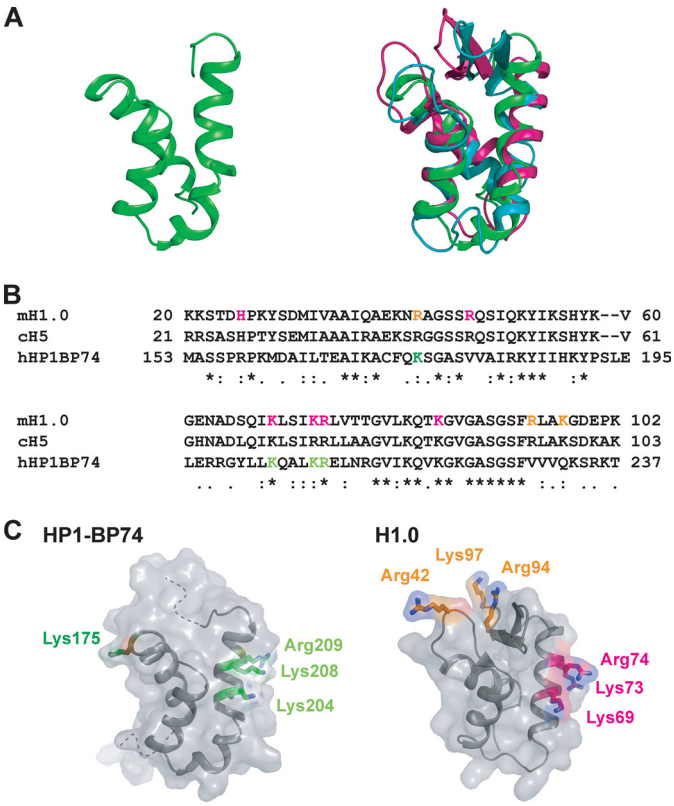

FIGURE 4. **Structure of the GD of HP1-BP74 (BP74GD).** *A*, three-dimensional structures of the BP74GD (*left*) and a superimposed image with chicken H1 GD (*cyan*) and chicken H5 GD (*magenta*) (*right*). *B*, sequence alignment of GD among mouse H1.0 (*mH1.0*), chicken H5 (*cH5*), and human HP1-BP74 (*hHP1-BP74*) by ClustalW2. As for the H1.0 sequence, the amino acids that form the larger binding site and the smaller binding site are *colored magenta* and *orange*, respectively (see *C*). The conserved residues are *colored light green* or *green* in HP1-BP74. *C*, the predicted nucleosome binding residues of HP1-BP74 (*left*) and H1.0 (*right*). The binding residues of H1.0 are mapped onto the atomic structure of the cH5 globular domain, as described previously (59).

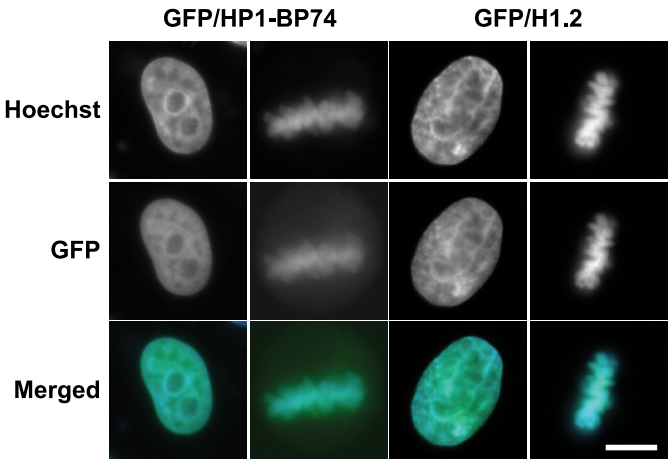

FIGURE 5. **Localization of GFP/HP1-BP74 in HeLa cells.** N-terminally GFP-fused full-length HP1-BP74 and H1.2 were transiently and stably expressed in HeLa cells, respectively. For the merged images, Hoechst and GFP signals are indicated in *blue* and *green*, respectively. *Bar*, 5 μm.

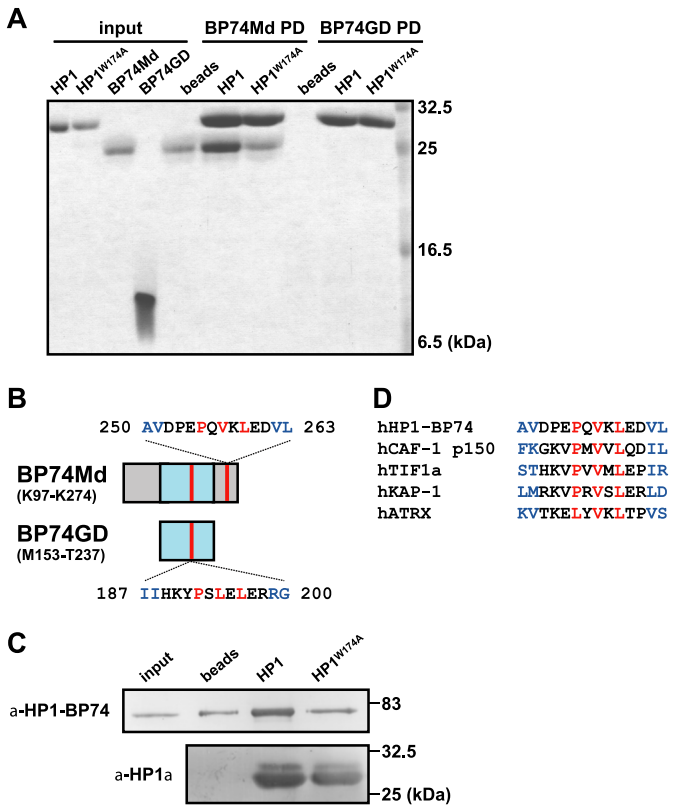

FIGURE 6. ***In vitro* binding of HP1-BP74 and HP1α.** *A*, His-tagged pull-down assay between HP1-BP74 and HP1α. HP1α with point mutations in CSD, C133A and W174A (*HP1^{W174A}*) and C133A (*HP1*), were assayed for binding to BP74Md or BP74GD. The bound proteins were visualized by CBB staining. The materials used for the assay were loaded as input. *B*, schematic diagrams of the recombinant BP74Md and BP74GD proteins. The GD is *colored light blue*. The *red bars* indicate the predicted P*X*V*X*L motifs, and corresponding amino acid sequences are shown. Consensus P*X*V*X*L motifs are *colored red*, and amino acids that might be involved in the interaction with HP1 are *colored blue*. *C*, His-tagged pull-down assay between the endogenous full-length HP1-BP74 and recombinant HP1α. HP1 and HP1^{W174A} were assayed for binding to the endogenous full-length HP1-BP74 prepared from the isolated chromosomes by 0.4 M NaCl stripping. The interaction assay was performed at 150 mM NaCl. The proteins were detected by Western blotting. For the detection of the recombinant HP1 proteins, one-thirtieth of the amount of the pull-down products used for the detection of HP1-BP74 was applied. *D*, comparison of the amino acid sequences responsible for HP1-binding among different proteins. The P*X*V*X*L motif is shown in *red*, and the additional residues involved in the interaction are shown in *blue*. *hCAF-1*, human chromatin assembly factor-1; *hTIF1*α, human transcriptional intermediary factor 1α; *hKAP-1*, human Krüppel-associated box (KRAB)-associated protein-1; *hATRX*, human α-thalassemia/mental retardation syndrome X-linked.

sequence located within BP74GD was previously reported to be a variant of the P*X*V*X*L motif and was essential for the binding to HP1α CSD (Fig. 6*B*) (44). However, in our current experiment, BP74GD containing only P*X*L*X*L did not co-precipitate with HP1α^{C133A}, suggesting that BP74Md possesses a P*X*V*X*L motif responsible for the interaction with HP1 in a region other than BP74GD. We found a P*X*V*X*L motif in the amino acid sequence of BP74Md at Ala^{250}–Leu^{263} (Fig. 6*B*). We also investigated the intermolecular interactions between BP74Md and HP1α^{C133A} using isothermal titration calorimetry. The obtained stoichiometry was 1:1, and the dissociation constant (K_d) was 2.4 μM (supplemental Fig. S5). We also investigated whether the endogenous full-length HP1-BP74 binds to HP1α. A 0.4 M NaCl-dissociated chromosomal protein fraction, which contained HP1-BP74, was employed for a pull-down assay using recombinant His-tagged HP1α proteins. As shown in Fig. 6*C*, the endogenous full-length HP1-BP74 was bound to HP1α but not to the W174A mutant, similarly to the BP74Md. According to these results, we concluded that one HP1-BP74 binds to one HP1α dimer through a newly identified P*X*V*X*L

motif located in the unstructured region outside the GD (Fig. 6*B*).

DISCUSSION

BP74Md Has Linker Histone-like Properties—In mammals, 11 H1 subtypes have been reported to date (49), namely H1.1–H1.5 (50, 51), H1.0 (52), H1.t (53), H1T2 (54, 55), H1Foo (56), HILS1 (57), and H1.X (58). Members of the H1 histone family have common biochemical and physical features that distinguish them from other chromatin proteins. They consist of three domains: a short unstructured highly basic N-terminal tail with a poorly defined function, a folded GD that is essential for binding to nucleosomes, and a long, highly basic C-terminal region. The GD binds to the nucleosome and interacts with either of the incoming/outgoing linker DNAs; the C terminus associates with both linker DNAs, leading to the formation of the stem structure in the chromatosome. In this study, we have demonstrated that BP74Md has linker histone-like properties in the sense that BP74GD within BP74Md has linker histone-like three-dimensional structure and that it binds to nucleosome at the entry/exit site of nucleosomal DNA. Some H1 variants are expressed in specific tissues (49), whereas HP1-BP74 expression is not restricted (see the Genevestigator Web site), suggesting that it contributes to chromatin structure and function globally. MNase digestion of the BP74Md-bound mononucleosome produced the chromatosome stop (Fig. 3*C*), suggesting that HP1-BP74 has a structural role similar to that of the linker histones through its BP74Md. In the present study, the similarity of three-dimensional structure between linker histone GD and BP74GD is elucidated (Fig. 4*A*). The localization pattern of linker histone and HP1-BP74 is also confirmed (Fig. 5). These results suggest a possible functional similarity between HP1-BP74 (or at least its middle region) and linker histones. Moreover, similar predicted nucleosome binding sites were observed between the GD of linker histone and BP74GD (Fig. 4, *B* and *C*, and supplemental Fig. S2*B*). The previous research on H1.0 revealed that the residues involved in nucleosome binding are spatially clustered to form two distinct binding sites, the larger site containing His^{25}, Arg^{47}, Lys^{69}, Lys^{73}, Arg^{74}, and Lys^{85} and the smaller site containing Arg^{42}, Arg^{94}, and Lys^{97} (59). The residues forming the large binding site are relatively conserved in HP1-BP74. Solution structure analysis revealed that Lys^{204}, Lys^{208}, and Lys^{209} are exposed to the solvent to form a basic patch, which corresponds to the larger binding site in H1.0 (Fig. 4*C*). However, the smaller binding site is not conserved in HP1-BP74. The absence of the smaller basic patch could cause weaker associations between BP74Md and DNA, consistent with the results of the chromatosome stop analysis in which BP74Md produced a less protected DNA fragment compared with that of H1.2 (Fig. 3*C*). The intrinsically disordered C terminus of linker histones forms the so-called stem structure, bridging the incoming and outgoing linker DNAs together (60). The interaction between the C terminus and the linker DNAs is believed to require an enrichment of positively charged amino acids in the C terminus. In case of HP1-BP74, the amino acid composition of C-terminal 133 residues, which is predicted as an intrinsically disordered region (supplemental Fig. S1), is also enriched in two basic amino acids (lysine and arginine): 28.6% in HP1-BP74 and 40.0% in H1.2. Thus, it is not surprising that HP1-BP74 and H1s are dissociated from the isolated chromosomes at the same NaCl concentration because they might interact with chromatin in a similar electrostatics-dependent manner.

Previously, we reported that H1.X has chromatin-binding activity and that its function is essential for proper mitotic progression (61). H1.X is functionally different from the other authentic H1 subtypes, and HP1-BP74 also appears to have distinctive functions other than the formation of the chromatosome structure. HP1-BP74 is much larger (553 amino acids) than the other linker histones (194–346 amino acids). We performed a 5% perchloric acid extraction of nuclei by which linker histones dissociate from chromatin (62). Surprisingly, no significant dissociation of HP1-BP74 was detected (supplemental Fig. S6). This result showed that HP1-BP74 was different from the linker histones in terms of the solubility in 5% perchloric acid, although BP74Md was found to have similar properties as those of H1 GD. The insolubility might be attributed to regions outside of BP74Md. It might be possible that HP1-BP74 possesses multiple functions *in vivo*. One of these additional functions is its interaction with HP1 proteins (Fig. 6, *A* and *C*), suggesting that HP1-BP74 cooperates with HP1 proteins.

HP1-BP74 Binds with HP1 through a PXVXL-CSD Interaction—We determined the exact HP1-binding site on HP1-BP74 to be P*X*V*X*L (Pro^{255}–Leu^{259}), which exists outside of the BP74GD (Fig. 6). Previously, P*X*L*X*L (Pro^{192}-Leu^{196}) in the BP74GD was reported as the binding motif for HP1 CSD (44); however, our pull-down analysis showed that HP1-BP74 does not bind to HP1α through the P*X*L*X*L (Fig. 6*A*). The previous study on the interaction between the CSD dimer and CAF-1 revealed that the CSD binding region containing P*X*V*X*L in CAF-1 is unstructured, and each residue of P*X*V*X*L faces the same surface and is surrounded by the HP1 dimer (47). However, our structural analysis of BP74GD revealed that Leu^{194} in P*X*L*X*L is completely buried in the molecule and forms hydrophobic interactions with the alkyl chain regions of Ile^{188} and Arg^{198} to stabilize the α-helix (supplemental Fig. S3*A*). Moreover, the P*X*L*X*L region of HP1-BP74 is completely involved in the formation of the short α-helix, resulting in a shorter relative distance between Pro^{192} and Leu^{196} compared with that of CAF-1. These structural properties further support our pull-down results. As for the P*X*V*X*L motif located outside of the BP74GD, it is probably unstructured. In fact, the CD spectra showed that the region forming secondary structures is limited to the GD in BP74Md (supplemental Fig. S2). Also, amino acid sequence analysis of HP1-BP74 indicates that the P*X*V*X*L motif is involved in the intrinsically disordered region (supplemental Fig. S1). This is also supported by the observation that the amino acid region in HP1-BP74 containing the P*X*V*X*L motif was rapidly digested by trypsin, which provided experimental identification of BP74GD (data not shown). Although P*X*V*X*L is essential for CSD binding, it was reported that additional flanking residues are also important for this interaction (47). Many of the CSD interaction partners contain hydrophobic residues at the −6/−7 and +5/+6 positions (Fig. 6*D*), which help stabilize the interaction. These features are conserved in BP74Md. Although the P*X*L*X*L motif in BP74GD also complies with this

pattern, I188 and R198 at the −6 and +5 positions, respectively, are involved in the intramolecular interaction and are not exposed to the solvent (supplemental Fig. S3*A*), strongly suggesting that the P*X*L*X*L motif in BP74GD is not a CSD binding site. These results obtained with recombinant proteins were also confirmed with the endogenous full-length HP1-BP74 (Fig. 6*C*), suggesting that the N- and C-terminal tails of HP1-BP74 do not disturb the interaction with HP1.

Thus, we conclude that we discovered a protein that not only shares some properties with linker histones but also binds to HP1. Further *in vivo* functional studies on HP1-BP74 are expected to provide new insight into the organization of the higher order structure of chromatin.

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